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Phagocytosis by haemocytes from the Lesser Octopus *Eledone cirrhosa*

Fagocitosis en hemocitos del pulpo blanco *Eledone cirrhosa*

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ABSTRACT

Haemocytes from *Eledone cirrhosa* phagocytose formalized bacteria (*Vibrio anguillarum*). The phagocytic capabilities of *E. cirrhosa* haemocytes are affected by several factors, including the haemocyte culture medium, temperature, duration of the assay, and the bacterial pre-incubation conditions such as haemolymph concentration, temperature and the duration of pre-incubation.

Haemocytes will phagocytose in the absence of haemolymph. With a 30min incubation period the number of phagocytosing haemocytes increases as the pre-opsonization concentration and incubation temperature increase. However after 2 hours at 15 or 20°C the number of haemocytes phagocytosing unopsonized bacteria is equivalent to the number engulfing 100% haemolymph opsonized bacteria.

RESUMEN

Los hemocitos de *Eledone cirrhosa* fagocitan bacterias formalizadas (*Vibrio anguillarum*). La capacidad de fagocitar en estas células se ve afectada por varios factores, incluyendo el medio de cultivo de los hemocitos, temperatura, duración del experimento, y las condiciones de preincubación de las bacterias, tales como concentración de hemolinfa y temperatura y duración de la preincubación. Los hemocitos fagocitan en ausencia de hemolinfa. Con un periodo de incubación de 30 minutos, el numero de hemocitos que fagocitan se incrementa cuando lo hacen la concentración de preopsonización y la temperatura de incubación. Sin embargo, tras dos horas a 15 ó 20°C, el número de hemocitos que fagocitan bacterias no opsonizadas es equivalente al de hemocitos que fagocitan bacterias tratadas con hemolinfa al 100%.

KEY WORDS: *Eledone cirrhosa*, haemocytes, phagocytosis, opsonization.
PALABRAS CLAVE: *Eledone cirrhosa*, hemocitos, fagocitosis, opsonización.

INTRODUCTION

In vivo and *in vitro* investigations into the cellular activities of molluscs have demonstrated that, in a number of cases, the blood cells or haemocytes are avidly phagocytic and capable of recognising non-self (reviewed by MILLAR AND RATCLIFFE,

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1994). The process of phagocytosis involves a number of recognizable stages, which include attraction, attachment, ingestion and killing of foreign organisms, and is influenced by a number of factors (reviews by RATCLIFFE, ROWLEY, FITZGERALD AND RHODES, 1985; MILLAR AND RATCLIFFE, 1994). Variables which have been shown to affect phagocytic rates in molluscs include incubation temperature (FOLEY AND CHENG, 1975), time and pH (ABDUL-SALAM AND MICHELSON, 1980), the size of the particle presented for phagocytosis and the nature of the particles (reviewed by BAYNE, 1983). Though phagocytosis will take place in the absence of opsonizing agents (RENWRANTZ AND STAHMER, 1983; TUAN AND YOSHINO 1987; FRYER, HULL AND BAYNE, 1989), several experiments have shown that soluble humoral factors or opsonins may be instrumental in non-self recognition (PROWSE AND TAIT, 1969) and, or enhancement of phagocytosis (reviews by JENKIN, 1976; RATCLIFFE *ET AL.*, 1985).

The haemocyte culture medium has been shown to influence phagocytosis with, in the case of the Asian clam, *Corbicula fluminea*, the presence of divalent cations being necessary for both opsonin-independent and opsonin-dependent phagocytosis (TUAN AND YOSHINO, 1987). The process of opsonization also appears to be influenced by several other factors. FRYER AND BAYNE (1989), using *Biomphalaria glabrata*, showed that for this mollusc opsonization is a time-dependent process. Further, TRIPP (1992), working with *Mercenaria mercenaria* demonstrated that at low temperatures, opsonization caused enhanced phagocytic rates.

The octopus *Eledone cirrhosa* is benthic in habit, ranges in depth from sub-littoral to 770 m and encounters temperatures between 5 and 15°C (BOYLE, 1983). The animal has a closed circulatory system and if wounded prevents blood loss by local vasoconstriction of the area surrounding the wound. The blood of the octopod does not clot and further blood loss is prevented by allowing seepage of blood through the wound until blood cells eventually plug the wound (WELLS, 1978, 1983; BAYNE, 1983). If the animal loses a large amount of blood a dilution of the respiratory pigment (haemocyanin) occurs which takes up to 2 hours to be reversed (WELLS AND WELLS, 1993). There appears to be only one main type of blood cell or haemocyte in *E. cirrhosa*. The haemocyte matures in the white body, or haematopoetic organ, of the animal and is released into the closed circulatory system (COWDEN AND CURTIS, 1974, 1981). Few cephalopod defense mechanisms have been elucidated (FORD, 1992). It is known that *E. cirrhosa* haemocytes will phagocytose erythrocytes only in the presence of haemolymph *in vitro* (STUART, 1968). Also *in vivo* studies (STUART, 1968; BAYNE, 1973) using different octopods, demonstrate that it is mainly fixed phagocytes in certain organs which clear injected foreign particles, with haemocytes only removing a small fraction of them.

This paper investigates whether haemocytes from *E. cirrhosa* are capable of phagocytosing dead bacteria *in vitro* and whether temperature, time and haemolymph concentrations influence phagocytosis. Additional experiments were also performed to determine whether bacterial pre-incubation (opsonization) at different temperatures, times and haemolymph concentrations affected phagocytic rates.

MATERIALS AND METHODS

Animals: Octopuses, *Eledone cirrhosa* (Lamarck) were obtained from crab pots around the North Wales coast. The animals were brought into the aquarium at the University of Bangor and maintained in natural seawater at 10-12°C. After 48 h the animals were weighed, marked using a panjet and assigned to a particular tank. Five octopuses per tank were chosen at random for each set of experiments.

Haemolymph: Blood was withdrawn from the branchial blood vessel of each octopus as described by MALHAM, SECOM-

BES AND RUNHAM (1995). The blood was centrifuged at 4°C for 5 min at 800g to remove the haemocytes. The resulting haemolymph from a number of individuals was pooled and frozen at -20°C. Before use the haemolymph was thawed and diluted to a final concentration of 0. 1, 1 or 10% in Sterile Octopus Saline (SOS) (NaCl, 2.367 g/100 ml; Glucose, 1 g/100 ml; $CaCl_2$, 0.116 g/100 ml; KH_2PO_4, 0.0056 g/100 ml; KCl, 0.1089 g/100 ml; $MgSO_4 \cdot H_2O$, 0.503 g/100 ml; $MgCl_2$, 0.419 g/100 ml).

Haemocytes: From each animal 1 ml blood samples were withdrawn into 10 ml of ice cold Marine Anticoagulant (NaCl, 2.63 g/100 ml; Glucose, 1.8 g/100 ml; Tri-Sodium Citrate, 0.088 g/ml; Citric Acid, 0.055 g/100 ml) containing ethylene glycol-bis(b-aminoethylether) N, N, N', N', -tetraacetic acid (EGTA) (0.029 g/100 ml). After a blood count the haemocytes were centrifuged at 800 g for 5 min at 4°C, and washed by resuspension in Octopus Ringer (NaCl, 2.433 g/100 ml; Glucose, 1.4 g/100 ml; EGTA, 0. 015 g/100 ml; KCl, 0.082 g/100 ml; KH_2PO_4, 0.004 g/100 ml) containing $CaCl_2$ (0.0142 g/100 ml), $MgCl_2$ (0.0524 g/100 ml) and $MgSO_4$ (0.0629 g/100 ml). A final haemocyte count was made before the haemocytes were washed for a second time and resuspended in SOS at 1×10^6 haemocytes/ml.

Bacteria: *Vibrio anguillarum* (MT275) were obtained from the Scottish Office, Agriculture and Fisheries Department, Marine Laboratory, Aberdeen. Formalized *V. anguillarum* were counted, washed twice by resuspension in SOS and centrifuged at 13000 g for 10 min before resuspension at 8×10^8 cells/ml in the required treatments.

Transmission electron microscope (T.E.M.) preparation: Five hundred μl of blood was withdrawn from the branchial blood vessel of the octopus and mixed directly with 500 μl of washed bacteria. After 2h incubation at 15°C the blood was centrifuged and the haemolymph removed. The pelleted haemocytes were fixed for 24 h at 4°C in 2.5% glutaraldehyde (in 0.1M sodium cacodylate buffer at pH 7.4). The haemocytes were washed in 0.1M sodium cacodylate buffer and secondarily fixed for 2 h at room temperature in 1% osmium tetroxide before staining *en bloc* with 2% uranyl acetate over night. The pellet was then dehydrated through ethanol and propylene oxide and embedded in Spurr resin. Cut sections (50 nm) were mounted on 100 mesh pioloform copper coated grids and stained with lead citrate. Sections were viewed in a GEC Corinth 500 at 60 KV.

Phagocytosis assay: Two phagocytosis experiments were performed to determine the effect of haemolymph concentration, temperature and time on haemocyte phagocytosis. Five animals were used for each experiment. The first experiment involved incubating haemocytes in 16 well tissue culture slides (Nunc) for 2 h at different temperatures, but utilizing one pre-incubation temperature and time for the bacteria. The second experiment involved haemocyte incubations of 30 min only and utilized different temperatures, times and haemolymph concentrations for bacterial pre-incubations.

For the first experiment 50 ml of the haemocyte suspension in SOS was put into each of the 16 well chambers of a tissue culture slide. Fifty microliters of either SOS or haemolymph diluted in SOS was added in duplicate, at half hour intervals, to selected wells. Bacteria were resuspended in either SOS or 100% haemolymph for 2 h at 15°C and washed twice before use. Fifty microliters of either SOS treated or haemolymph treated bacteria immediately followed the haemolymph additions, again in duplicate. Each well of the tissue culture slide therefore contained: 50 ml of haemocytes in SOS, 50 ml of either SOS or haemolymph diluted in SOS to 0.1, 1 or 10% concentration (final concentrations of 0.03, 0.33 or 3.33% respectively) and 50 ml of bacteria resuspended in SOS after treatment. The assays were run at four temperatures (5, 10, 15 and 20°C). After 2 h the tissue

culture slides were rinsed in SOS to remove unattached bacteria and the slide fixed by immersion in methanol for 3-5 min.

The second experiment involved the addition of 50 ml of haemocytes in SOS at 1 x 10^6 haemocytes/ml, followed by 50 ml of haemolymph diluted in SOS at 0, 0. 1, 1 or 10% concentrations and 50 ml of the different bacterial preparations added in duplicate to the tissue culture slides. The bacteria were washed and resuspended in haemolymph at concentrations of 0, 0.1, 1, 10 or 100%, using Phosphate Buffered Saline pH 7.0 (PBS, Gibco, without Ca^{2+} and Mg^{2+}) as the diluent. Bacteria were incubated for 1, 10, 60 or 120 min at 5, 10, 15 or 20°C, before being washed twice and used in the assay. The slides were incubated at temperatures of 5, 10, 15 or 20°C. After 30 min the tissue slides were rinsed with SOS and the experiment stopped by immersion of the slide in methanol as previously.

All slides were then stained in Giemsa (Sigma), rinsed in Gurr Buffer (BDH pH 6.8) and air dried before mounting using DPX.

Statistical analysis: Analysis was performed by random counting of 200 haemocytes in each well. The haemocytes were counted under oil using a compound binocular microscope at 800x magnification. All slides were numbered and randomly selected to reduce observer bias. The number of haemocytes which had phagocytosed bacteria was expressed as a percentage of the haemocytes counted in each of the duplicate wells. The results for each of the duplicate wells were averaged and analysis of variance (ANOVA) performed for the 2 experiments using the 5 replicates. In each case P values of < 0.05 were taken as being significant. The replicate means were calculated and Tukey's pairwise comparison was performed for each experiment using the calculated confidence interval estimation (CI estimation). The CI estimate allows 2 separate means to be statistically compared (Rice, 1988).

RESULTS

Phagocytosis of the formalized Gram negative bacterium, *V. anguillarum*, by *E. cirrhosa* haemocytes occurs both in the presence and absence of haemolymph. Collected haemocytes were incubated with bacteria for 2 h before fixation for T.E.M. Sections clearly indicate that *E. cirrhosa* haemocytes phagocytose and degrade bacteria (Fig. 1).

From Analysis of variance a number of significant conclusions were obtained. Phagocytosis by haemocytes following pre-incubation of the bacteria in 100% haemolymph was significantly greater than phagocytosis following SOS treatment ($F= 594.85$, $P<0.0001$) (Fig. 2). Highly significant values were also obtained for the effect of incubation temperature ($F= 155.09$, $P<0.0001$), and also for the duration of the assay ($F= 178.9$, $P<0.0001$). The concentrations of haemolymph used in the assay medium did not have a significant effect ($F= 0.32$, $P= 0.814$) indicating that the rate of phagocytosis was statistically equivalent in assays containing 0, 0.1, 1 or 10% haemolymph.

Cross-wise comparisons of the percentage of haemocytes phagocytosing opsonized and unopsonized bacteria, temperature and assay duration were also highly significant, ($P<0.0001$), whereas cross-wise comparisons involving haemolymph concentration in the assay medium, confirmed that the haemolymph concentrations, in SOS, did not affect phagocytic rates. Haemolymph concentration was therefore not considered in further analysis, and results at each temperature and time were pooled.

Phagocytosis of bacteria pre-incubated in SOS was affected by temperature and time (Fig. 2A). At all temperatures the number of haemocytes engulfing bacteria increased over time. At 20°C there appeared to be fewer haemocytes phagocytosing than at 15°C, however statistically there was no difference between the means at the 2 temperatures. At 10°C there was a rapid increase in the number of haemocytes phagocy-

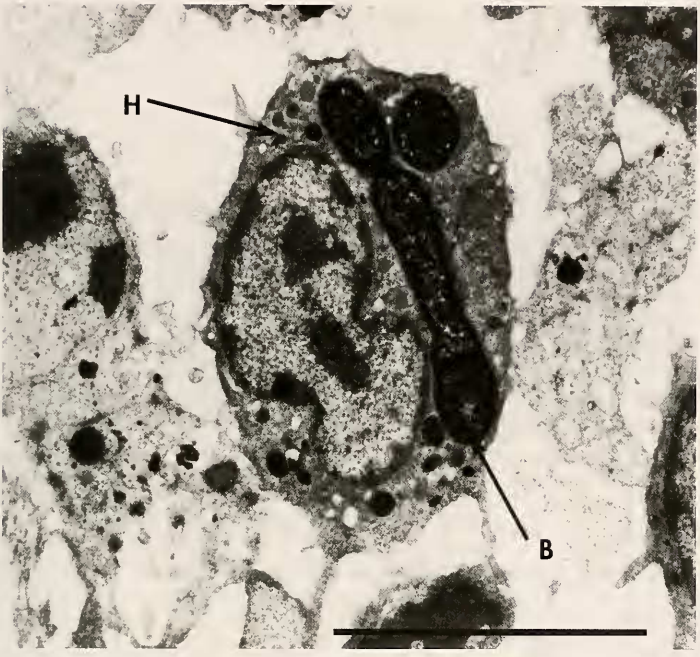

Figure 1. Transmission electron micrograph of an *Eledone cirrhosa* haemocyte (H) having engulfed a bacterium (*Vibrio anguillarum*) (B). Scale bar 10 µm.
Figura 1. Microfotografía de un hemocito (H) de Eledone cirrhosa *tras haber tragado una bacteria (*Vibrio anguillarum*) (B). Escala 10 µm.*

tosing bacteria during the first 30 min followed by a slower rate of increase up to 2 h. At both 5 and 10°C significantly lower phagocytic rates were observed than at 15 and 20°C over the 2 h period. Fig. 2B shows the mean number of haemocytes phagocytosing bacteria, pre-incubated in 100% haemolymph, over time. The haemocyte phagocytic rate again increased over the 2 h period but there were far smaller differences between the incubation temperatures. The phagocytic rates were again lower at 5°C than at the other temperatures. The maximum increase in phagocytosis at all temperatures occurred within the first 30 min.

As with the first experiment, the different concentrations of haemolymph in SOS (at 0, 0.1, 1 or 10%) used in the second assay were found to have little effect, so were removed from the pair wise comparison with no appreciable percentage error increase (0.027%) and the results pooled at each pre-incubation temperature and time. To simplify the pairwise comparison the assay temperature was not included as a main factor, but was added as an interacting factor. The results from the simplified model show that there were large statistically significant differences ($F= 1083.35$, $P<0.0001$) between the haemolymph pre-incubation concentrations. The pre-incubation temperatures ($F= 61.32$, $P<0.0001$), and the pre-incubation times ($F= 725.24$, $P<0.0001$) were similarly significantly different. Pre-incubation of the bacteria in PBS alone at different temperatures and time periods caused no significant difference in the phagocytic rate (Fig. 3). Bacteria pre-incubated in 0.1% haemolymph in PBS at all pre-incubation temperatures and times were phagocytosed at a significantly lower rate than in PBS alone. Pre-incubation of the bacteria

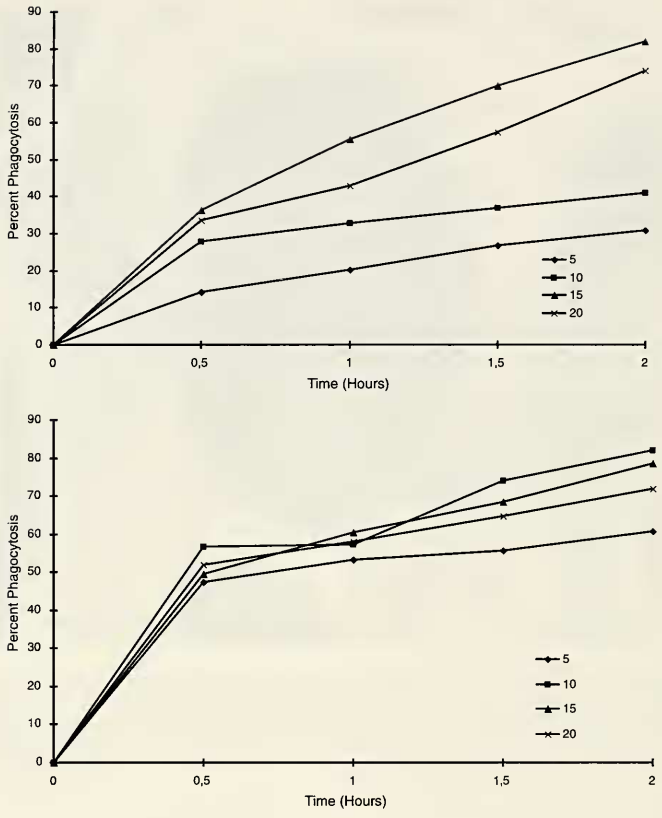

Figure 2. A: phagocytosis of non-opsonized formalized *Vibrio anguillarum* at 4 temperatures over a 2h haemocyte incubation period. The bacteria were pre-treated with SOS for 2h at 15°C. Tukeys CI estimate= 9.52. B: phagocytosis of opsonized formalized *Vibrio anguillarum* at 4 temperatures over a 2h haemocyte incubation period. The bacteria were pre-treated with 100% haemolymph for 2 h at 15°C. Tukeys CI estimate = 9.52.

Figura 2. A: fagocitosis de bacterias formalizadas Vibrio anguillarum *no opsonizadas a cuantro temperaturas sobre un periodo de incubación de hemocitos de dos horas. Las bacterias fueron pretratadas con SOS durante 2 horas a 15°C. Estimación CI de Tukeys= 9,52. B: fagocitosis de* Vibrio anguillarum *formalizado y opsonizado a cuatro temperaturas sobre un periodo de incubación de hemocitos de 2 horas. Las bacterias fueron pretratadas con hemolinfa al 100% durante 2 horas a 15°C. Estimación del intervalo de confianza de Tukeys= 9,52.*

in 1% haemolymph showed initially the same lowered phagocytic rate as for 0.1% pre-incubation. However, pre-incubation of the bacteria in 1% haemolymph for 10 min at 20°C caused an enhanced phagocytic rate which also occurred at all temperatures at 60 and 120 min. Bacteria pre-incubated in 10% haemolymph for 1 min at 5, 10, 15 and 20°C and for 10 min at 5 and 10°C were statistically equivalent to the values determined in PBS alone. However, at 10 min following pre-incubation at 15 and 20°C more haemocytes were observed phagocytosing bacteria than at 5 or 10°C, or at 1 min at all temperatures. The enhanced phagocytic rate observed using 10% haemolymph is statistically equivalent to the enhanced rate observed at 1% concentration.

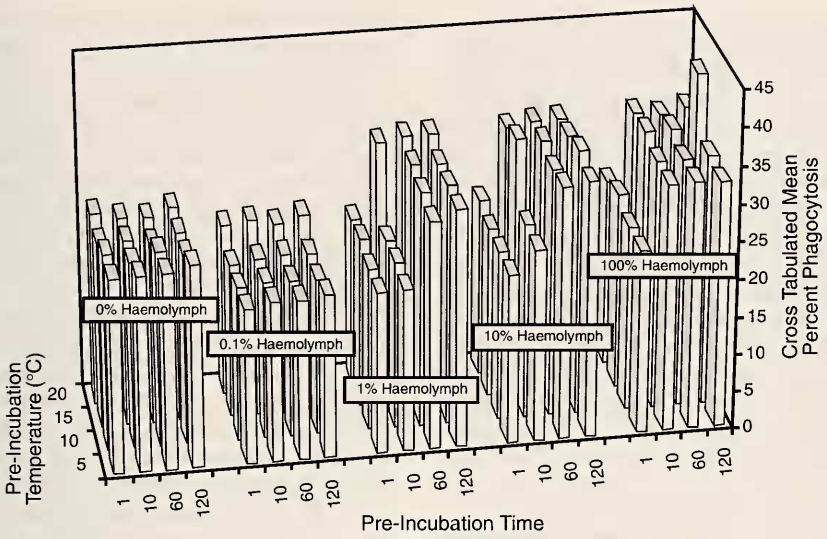

Figure 3. Phagocytosis of formalized *Vibrio anguillarum*. The haemocytes were incubated at different temperatures for 30 min only. The bacteria were pre-incubated in 0% haemolymph (*i.e.*, PBS only), 0.1% haemolymph, 1% haemolymph, 10% haemolymph and 100% haemolymph concentrations. The bacterial pre-incubation temperatures were 5, 10, 15 and 20°C and the pre-incubation times were 1, 10, 60 and 120 min. Tukeys CI estimate = 3.1.

Figura 3. Fagocitosis de Vibrio anguillarum *formalizado. Los hemocitos fueron incubados a diferentes temperaturas durante sólo 30 minutos. Las bacterias fueron preincubadas en concentraciones de hemolinfa del 0% (*i.e., *sólo PBS), 0,1%, 1%, 10% y 100%. Las temperaturas de preincubación de las bacterias fueron 5, 10, 15 y 20°C y los tiempos de preincubación de 1, 10, 60 y 120 minutos. Estimación del intervalo de confianza de Tukeys= 3,1.*

DISCUSSION

The results presented here demonstrate that *E. cirrhosa* haemocytes are capable of recognizing and ingesting the formalized bacterium *Vibrio anguillarum*. *V. anguillarum* is a Gram negative commensal marine opportunist and was chosen as the experimental bacterium because it has been isolated from, and used in previous studies on wound healing in *E. cirrhosa* (BULLOCK, POLGLASE AND PHILLIPS, 1987). This bacterium has also been implicated in causing cephalopod infections when the animals are held in captivity and is a common contributory cause of death at high aquarium temperatures (LEIBOVITZ, MEYERS AND ELSTON, 1977; HANLON, FORSYTHE, COOPER, DINUZZO, FOLSE AND KELLY, 1984; FORD, ALEXANDER, COOPER AND HANLON, 1986; HANLON AND FORSYTHE, 1990).

STUART (1968) found that *E. cirrhosa* haemocytes required haemolymph for *in vitro* phagocytosis of erythrocytes. The data presented in this paper demonstrate that the presence of haemolymph is not necessary for ingestion of bacteria. However, this bacterium is smaller with far less surface area than an erythrocyte and as such maybe more easily phagocytosed. It was found by TYSON AND JENKIN (1974) that haemocytes from a crayfish (*Parachaeraps bicarinatus*) phagocytosed bacteria in the absence of haemolymph, but erythrocytes were not phagocytosed unless they were pre-treated with haemolymph (MCKAY, JENKIN AND ROWLEY, 1969). Further JENKIN (1976), suggested that the concentration of certain recognition

molecules on the crayfish haemocyte surface was not sufficient to bind erythrocytes, but was sufficient to bind bacteria, and a similar explanation could apply to *E. cirrhosa* haemocytes. Another possibility was demonstrated by BAYNE, MOORE, CAREFOOT AND THOMPSON, (1979), who showed that haemocytes from *Mytilus californianus* had a greater affinity for yeast cells than human erythrocytes, and suggested that phagocytosis of foreign particles was selective. Results from other molluscan species also demonstrate that surface antigenicity of the respective test particles has an effect on phagocytosis by haemocytes (TRIPP AND KENT, 1967; ANDERSON AND GOOD, 1976).

TRIPP (1966), using the bivalve *M. mercenaria*, concluded that haemolymph pre-treatment of erythrocytes caused increased phagocytosis. The same experiment showed however that if untreated erythrocytes were incubated with haemocytes for longer periods of time, the same levels of phagocytosis were achieved. With *E. cirrhosa* haemocytes at 15 and 20°C the phagocytic rate is higher at 30 min for 100% haemolymph treated bacteria compared to SOS treated bacteria, but after 2 h there was no difference in phagocytic rates between the 2 treatments. The data presented here also indicate that a higher percentage of haemocytes phagocytosed haemolymph treated bacteria at 5 and 10°C over 2 h than SOS treated bacteria. TRIPP (1992) also showed that the haemocytes of *M. mercenaria* were avidly phagocytic in the absence of haemolymph, however at low temperatures, in the presence of haemolymph there was increased phagocytosis of yeast. ABDUL-SALAM AND MICHELSON (1980), working with *Biomphalaria glabrata*, also demonstrated that temperature has an effect on haemocyte phagocytosis. A phagocytic activity peak was evident at 30°C with inhibition of phagocytosis below 15°C. Low temperature inhibition (4°C) of phagocytic rates has also been demonstrated for the haemocytes from the hard clam *M. mercenaria* with maximum rates occurring at 22 and 37°C (FOLEY AND CHENG, 1975). With SOS treated bacteria, *E. cirrhosa* haemocytes demonstrate an activity peak with about 70% of haemocytes phagocytosing after 2 h at 15 and 20°C. At 5°C only 14% of haemocytes contained bacteria, whereas if the bacteria were initially pre-incubated in haemolymph before addition to the assay the phagocytic rate at 5°C increased to around 47%.

The results presented above indicate that the amount of haemolymph present in the bacterial pre-incubation medium has a dramatic effect on the number of haemocytes subsequently engulfing these bacteria within a 30 min period. Haemolymph concentrations of 0.1 and 1% in PBS, resulted in lower numbers of haemocytes phagocytosing compared to PBS alone. This inhibition changes to enhanced phagocytosis, at all higher pre-incubation concentrations. Further comparisons demonstrate that the temperature of the pre-incubation medium and particularly the duration of incubation are also important factors. The observed trends indicate that increasing the pre-incubation temperature decreases the pre-incubation time needed for enhanced phagocytosis to occur. FRYER *ET AL.* (1989), working on *B. glabrata*, similarly demonstrated that phagocytosis was inhibited after short pre-incubation periods, whereas longer pre-incubation periods of 1 h resulted in enhanced levels. It was suggested by the authors that initial non-specific adsorption of a variety of plasma components (opsonins) occurred onto, in their case, the yeast surface. Longer exposure to the plasma allowed more of the opsonins to bind to the yeast surface. The results from the data presented here for the different pre-incubation haemolymph concentrations and durations of exposure seem to support this hypothesis. In addition it is possible that if the temperature is increased further more of the available plasma components would adhere onto the surface of the bacterium.

When haemocytes from *E. cirrhosa* were resuspended in SOS, as stated above, there is phagocytosis of the for-

malized bacterium *V. anguillarum*. In buffers containing either EDTA or EGTA, no phagocytosis of the same bacterium was evident (Malham, unpublished data). SOS contains Ca^{2+} and Mg^{2+} and it appears likely that the presence of these divalent ions has an effect on phagocytosis. FRYER AND ADEMA (1993) showed that manipulated haemocytes from *B. glabrata* retained some phagocytic activity, but that addition of excess Ca^{2+} and Mg^{2+} to the haemocytes before the addition of the target particles enhanced their phagocytic rates. *E. cirrhosa* haemocytes were initially drawn into an anticoagulant buffer containing EGTA and washed in Octopus Ringer, also containing EGTA, before resuspension in EGTA-free-SOS, all of which could alter haemocyte behaviour and affect phagocytosis. *Corbicula fluminea* haemocytes (TUAN *ET AL.*, 1987) also required extracellular Ca^{2+} or Mg^{2+} for both opsonin-dependent and independent phagocytosis. The authors suggest that the opsonin possibly exists as a divalent cation-macromolecular complex due to the loss of enhanced phagocytosis after dialysis against EDTA and EGTA. Further, *Mytilus edulis* haemocytes phagocytosed yeast cells with high efficiency when calcium ions were present in the suspension medium, and gave similar results when haemolymph alone was added, but almost no phagocytosis was recorded with haemocytes in buffered saline (RENWRANTZ AND STAHMER, 1983). When *V. anguillarum* was resuspended in SOS, *E. cirrhosa* haemolymph diluted in SOS, or in PBS alone, there was no change in the haemocyte phagocytic rate. However, when *V. anguillarum* was resuspended in haemolymph diluted in PBS (≥ 1% haemolymph concentration) or in haemolymph alone, enhanced phagocytosis was observed.

Haemolymph lectins have been shown to act as opsonins for haemocyte phagocytosis (e. g., RENWRANTZ, 1983; RENWRANTZ 1986; SMINIA AND VAN DER KNAPP, 1986, VASTA, 1991). Agglutination results from *Octopus maya* (FISHER AND DINUZZO, 1991) further support the role of lectins in recognition of non-self. Studies using the molluscs *Mytilus edulis* (RENWRANTZ AND STAHMER, 1983) and *Lymnaea stagnalis* (VAN DER KNAPP, 1982) have demonstrated that molecules antigenically related to haemolymph lectins have been found in the cytoplasm and on the surface of haemocytes. Lectins, in particular C-type, are found in a number of invertebrates including *Octopus vulgaris*. These lectins are Ca^{2+} dependent, and these ions are required for ligand binding of the lectin (RÖGENER, RENWRANTZ AND UHLENBRUCK, 1986). STUART (1968) suggested a possible link between an opsonic factor and haemocyanin in *E. cirrhosa*. Also a lectin identified from the haemolymph of *O. vulgaris* has been shown to be similar to a haemocyanin subunit (RÖGENER, RENWRANTZ AND UHLENBRUCK, 1985). The nature of the soluble factor causing enhanced phagocytosis in *E. cirrhosa* has not been studied, however the factor(s) must be present at a high concentration, since it is effective at a haemolymph concentration of 1% at 15 and 20°C.

In conclusion, *in vitro* phagocytosis of *Vibrio anguillarum* by haemocytes from *E. cirrhosa* is aided by a component of haemolymph and is affected by temperature, duration of the assay and preincubation of the bacterium with different haemolymph concentrations. Further studies to elucidate whether *E. cirrhosa* haemocytes are capable of phagocytosing and digesting live microorganisms *in vitro* and *in vivo* are being pursued.

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